

Purification of DNA Complementary to Nucleotide Sequences Required for Neoplastic Transformation of Fibroblasts by Avian Sarcoma Viruses

DOMINIQUE STEHELIN†, RAMAREDDY V. GUNTAKA, HAROLD E. VARMUS
AND J. MICHAEL BISHOP

*Department of Microbiology
University of California
San Francisco, Cal. 94143, U.S.A.*

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We have prepared radioactive DNA (cDNA_{sarc}) complementary to nucleotide sequences which represent at least a portion of the viral gene(s) required for neoplastic transformation of fibroblasts by an avian sarcoma virus. The genetic complexity of cDNA_{sarc} (~1600 nucleotides) is sufficient to represent an entire cistron. The genomes of three independent isolates of avian sarcoma viruses share nucleotide sequences closely related to cDNA_{sarc}, whereas the sequences are absent from transformation-defective mutants of avian sarcoma viruses, several avian leukosis viruses, a non-pathogenic endogenous virus of chickens (Rous-associated virus-O), sarcoma-leukosis viruses of mice and cats, and mouse mammary tumor virus. We conclude that the transforming gene(s) of all avian sarcoma viruses have closely related or common genetic lineages distinct from the transforming genes in sarcoma viruses of other species. Our results conform to previous reports that transformation-defective variants of avian sarcoma viruses are mutants with identical regions deleted from each subunit of a polyploid genome.

1. Introduction

Infection of fibroblasts by certain strains of RNA tumor viruses can lead to neoplastic transformation of the host cells (Temin, 1971). Genetic analyses have implicated specific viral genes in the transforming process (Martin, 1970; Kawai & Hanafusa, 1971; Bader, 1972), but the nature of these genes and the means by which their expression is regulated are not known. To facilitate investigation of these issues, we have prepared radioactive DNA complementary to nucleotide sequences which represent at least a portion of the viral gene(s) required for transformation of fibroblasts by AS virus†; we have denoted this specific DNA as cDNA_{sarc}.

Our procedure to isolate cDNA_{sarc} exploits the existence of genetic variants of AS viruses which cannot induce sarcomas in animals or transform fibroblasts in culture:

† Present address: I.R.S.C., B.P. 8, 94800-Villejuif, France.

‡ Abbreviations used: AS virus, avian sarcoma virus; td, transformation defective; Pr-C, Prague strain-subgroup C; B77-C, Bratislava 77-subgroup C; cDNA_{sarc}, single-stranded DNA complementary to nucleotide sequences found in the genome of AS viruses but not in the genome of td segregants; cDNA_{B77}, single-stranded DNA complementary to part or all of the genome of B77-C AS virus.

these transformation-defective strains are deletion mutants which are segregated during propagation of cloned AS viruses (Duesberg & Vogt, 1970, 1973*a,b*; Vogt, 1971; Martin & Duesberg, 1972) and which lack 10 to 20% of the genetic information in the parental virus (Duesberg & Vogt, 1973*b*; Lai *et al.*, 1973; Neiman *et al.*, 1974). Single-stranded DNA complementary to the genome of AS virus was prepared by standard techniques, using the RNA-directed polymerase activity of detergent-activated virions (Temin & Baltimore, 1972). DNA specific for the nucleotide sequences deleted from the genome of a td variant was then isolated by exhaustive hybridization with RNA from the td strain and the parental AS virus. The present communication describes the details of this preparation, documents some physical and chemical properties of cDNA_{sarc}, and explores the distribution of nucleotide sequences homologous to cDNA_{sarc} among various strains of RNA tumor viruses.

2. Materials and Methods

(a) Reagents and solutions

Reagents were obtained as follows: hydroxylapatite from BioRad; actinomycin D from Calbiochem; deoxynucleoside triphosphates from Schwartz-Mann; carrier-free [³²P]orthophosphate and [α -³²P]TTP (50 to 150 Ci/mmol) from International Chemical and Nuclear Corp; [³H]TTP (57 Ci/mmol) from Schwartz-Mann; RNase A from Worthington Biochemicals; Nonidet-P40 from Shell Chemical Co; diethylpyrocarbonate from Sigma Chemical Co. SI nuclease was prepared as described by Sutton (1971). Sodium phosphate buffers, pH 6.8, were prepared by mixing equal parts of Na₂HPO₄ and NaH₂PO₄. Solutions were treated with diethylpyrocarbonate (0.1% v/v) to inactivate contaminating nucleases.

(b) Cells and viruses

Viruses were propagated in chick embryo fibroblasts as described previously (Bishop *et al.*, 1970). Cloned Pr-C AS virus was grown from single colonies of transformed chicken cells provided by P. Vogt. Pr-C AS virus was also obtained as concentrated suspensions from University Laboratories Inc., Highland Park, N.J. (through the auspices of the Office of Program Resources and Logistics, National Cancer Institute). Approximately 50% of the virus from University Laboratories consisted of td variants, whereas the Pr-C AS virus from P. Vogt contained no detectable td particles when measured by molecular hybridization (unpublished results). A high titer isolate of B77-C AS virus was originally obtained from R. Friis; after repeated passage in our laboratory, this virus now consists mainly of td variants (~90% of the particles; unpublished results) but retains a high titer of transforming virus (>10⁷ focus-forming units per ml). Transformation-defective variants of Pr-C and B77-C AS virus, isolated as described previously (Vogt, 1971), were obtained from P. Vogt; both variant strains have been shown to be deletion mutants (Duesberg & Vogt, 1973*a,b*; Lai *et al.*, 1973).

Other viruses were obtained as follows: the Schmidt-Ruppin and Bryan strains of AS virus from H. Rubin; the Carr-Zilber and Fujinami strains of AS virus, Rous-associated virus-2, Rous-associated virus-0, Rous-associated virus-6, Rous-associated virus-50, and avian myeloblastosis virus from P. Vogt; ring-necked pheasant virus from D. Fujita (this is a non-transforming virus which is probably a recombinant between an endogenous virus of ring-necked pheasants and the Bryan strain of AS virus, see Fujita *et al.*, 1974); Moloney strain of murine sarcoma-leukemia virus and the Rickard strain of feline sarcoma-leukemia virus from Electronucleonics Inc.; murine mammary tumor virus (purified from the milk of tumor-bearing RIII mice) from N. Sarkar and D. Moore; and visna virus from A. Haase. Viruses were received as purified concentrates or were purified from culture medium as described previously (Bishop *et al.*, 1970). For preparation of viral RNA, virus was frequently extracted after sedimentation into a pellet (19,000 revs/min,

90 min, 4°C, Spinco L19 rotor); this procedure, performed directly on culture media after clarification (9000 g, 10 min, 4°C), provided satisfactory yields of viral RNA and obviated the need for more extensive purification of the virus.

(c) *Preparation of virus-specific DNA*

Reaction mixtures contained Pr-C AS virus (~2.0 mg protein/ml); 0.1 M-Tris-HCl (pH 8.1), 8 mM-MgCl₂, 2% (v/v) β-mercaptoethanol, actinomycin D (100 µg/ml), unlabeled dATP, dCTP and dGTP (each 2×10^{-4} M), [³H]dTTP (5×10^{-6} M; 0.3 mCi/ml), and Nonidet-P40 (0.08% v/v). The reaction mixture was supplemented with unlabeled dATP, dCTP and dGTP (to 4×10^{-4} M) at 4 h, and incubation continued for 18 h at 37°C. The enzymatic product was extracted and fractionated into single and double-stranded DNA by elution from hydroxylapatite as described previously (Fanshier *et al.*, 1971). The eluates were treated with 0.3 N-NaOH (37°C 2 h), neutralized, and DNA recovered by precipitation with cetyltrimethylammonium bromide. The DNA had a specific activity of 2×10^7 cts/min per µg, computed from the specifications for the isotope furnished by the supplier. Single-stranded DNA complementary to the genome of B77-C AS virus was prepared in an identical manner, using [³²P]dATP at 2×10^{-6} M (0.25 mCi/ml); the specific activity of the DNA was 2×10^7 to 10×10^7 cts/min per µg when used.

RNA was extracted from virus as described previously (Quintrell *et al.*, 1974); the organic extractant consisted of phenol and chloroform (mixed in a ratio of 2:1). Viral RNA was separated into its 70 S and low molecular weight constituents by rate-zonal centrifugation (Bishop *et al.*, 1970). To prepare high molecular weight subunits (~35 S) of the viral genome (Duesberg, 1968; Erikson, 1969), virus was harvested at intervals of 6 h; 70 S RNA was purified, denatured in 97% dimethylsulfoxide (25°C for 30 min), and fractionated by rate-zonal centrifugation (Bishop *et al.*, 1970). ³²P-labeled viral RNA was prepared at specific activities of 1×10^7 to 3×10^7 cts/min per µg as described previously (Quintrell *et al.*, 1974).

(d) *Molecular hybridization*

Reactions were carried out in either 0.6 M or 0.9 M-NaCl, containing 0.02 M-Tris-HCl (pH 7.4) and 0.01 M-EDTA. Hybridization of DNA was measured by hydrolysis with S1 nuclease as described previously (Leong *et al.*, 1972), and hybridization of RNA by hydrolysis with RNase A (50 µg/ml in 0.3 M-NaCl, 0.03 M-sodium citrate, 37°C, 45 min) (Quintrell *et al.*, 1974). Nucleic acids were prepared for scintillation counting by precipitation with perchloric acid and collection on glass fiber filters. Results of hybridization were expressed as a function of C_0t (concentration of DNA (mol/l) × time (s); see Britten & Kohne, 1968) or $C_t t$ (concentration of RNA (mol/l) × time (s); see Leong *et al.*, 1972), corrected to standard conditions (Britten & Smith, 1970).

(e) *Chromatography on hydroxylapatite*

Suspensions of hydroxylapatite (1 g in 5 ml 0.01 M-sodium phosphate) were boiled for 10 min prior to use and columns formed by pouring the equivalent of about 0.5 ml packed volume of hydroxylapatite into a pipette plugged with glass wool. The columns were maintained at 60°C with a circulating water bath. Samples were loaded in 0.01 M-sodium phosphate, followed by washing with the same buffer. A linear gradient (10 ml total) of sodium phosphate (0.01 M to 0.16 M) was then applied to the hydroxylapatite column with a peristaltic pump (flow rate approx. 0.4 ml/min); 0.8-ml fractions were collected. At the end of the gradient, the column was washed with 0.4 M-sodium phosphate. A portion of each fraction was analyzed for radioactive material by precipitation with perchloric acid. The phosphate concentration was determined by measurement of refractive index.

(f) *Precipitation of nucleic acids with cetyltrimethylammonium bromide*

Nucleic acids were recovered from phosphate buffers by a slight modification of the procedure described by Reitz *et al.* (1972). After addition of nuclease-free carriers (10 µg of heat denatured calf thymus DNA/ml and 100 µg of yeast RNA/ml), the nucleic acids were precipitated by adjusting the solution to 5 mM-cetyltrimethylammonium bromide. The suspension was kept at 0°C for 10 min, and centrifuged for 10 min at 16,000 g. Traces

of labeled DNA still remaining in solution were precipitated by a new addition of 10 μ g yeast RNA/ml in the same tube, without resuspending the pellet. Following a second centrifugation (10 min at 16,000 g), the pellet was resuspended in 1 M-NaCl. NaOH was then added to 0.3 N and the solution kept at 37°C for 2 h in order to eliminate RNA and possible traces of ribonuclease; the solution was neutralized with HCl and the nucleic acids precipitated by 3 vol. ethanol at -20°C for 6 h or more. Sodium chloride, which inhibits precipitation of nucleic acids by cetyltrimethylammonium bromide, could be eliminated by preliminary precipitation of nucleic acid and phosphates with 3 vol. ethanol. The pellet was then resuspended in 0.01 M-Tris-HCl (pH 7.4) and the precipitation with cetyltrimethylammonium bromide carried out as described.

(g) *Thermal denaturation of RNA/DNA hybrids on hydroxylapatite*

Hybrids were formed with 70 S RNAs of AS virus (1 μ g) and a mixture of [3 H]cDNA_{sarc} (3000 cts/min, 0.15 ng) and [32 P]cDNA_{B77} (5000 cts/min, 0.2 ng) in buffer containing 0.6 M-NaCl at 68°C (final C_{pH} of >100 mol-s/l). The hybrids in 0.12 M-phosphate buffer at room temperature were loaded on to columns of hydroxylapatite which were then washed continuously with 0.12 M-sodium phosphate (0.3 ml/min) while the temperature of the column was raised in increments of 2 deg.C every 3.3 min. The wash buffer was preheated to column temperature. Fractions (1-ml) were collected and analyzed for acid-precipitable radioactivity eluted as a consequence of the thermal gradient.

3. Results

(a) *Strategy for the preparation of cDNA_{sarc}*

The general strategy for isolation of cDNA_{sarc} is presented in Figure 1. Single-stranded DNA complementary to the genome of AS virus is prepared by conventional techniques and annealed to RNA from a td variant; the DNA which fails to anneal, representing viral nucleotide sequences absent from the genome of the deletion mutant, should be separable from RNA/DNA hybrids by chromatography on hydroxylapatite.

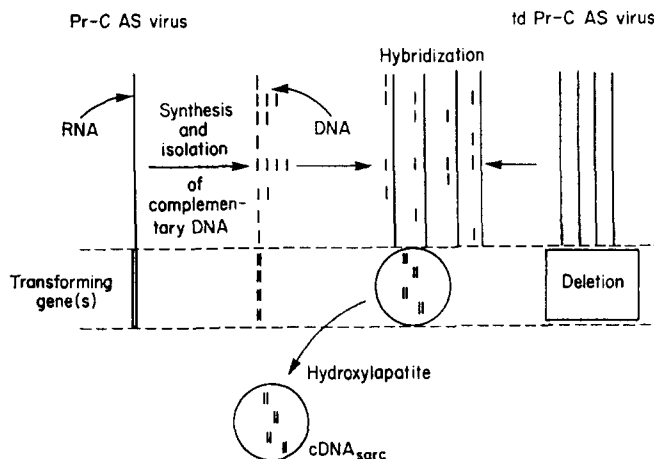


FIG. 1. Strategy for the preparation of cDNA_{sarc}.

Radioactive DNA complementary to the genome of AS virus was synthesized with detergent-activated virions, released from template RNA by hydrolysis with RNase, purified by chromatography on hydroxylapatite, and then annealed with a large excess of 70 S RNA from the deletion mutant td Pr-C AS virus. DNA which failed to anneal (cDNA_{sarc}) was purified by chromatography on hydroxylapatite and should correspond to the nucleotide sequences deleted from the genome of the parental sarcoma virus in the genesis of the td variant. As explained in the text, several modifications of this procedure proved necessary to effect satisfactory purification of cDNA_{sarc}.

In practice, we found it necessary to modify this scheme in order to circumvent several major difficulties.

(i) *High resolution chromatography on hydroxylapatite*

We anticipated that cDNA_{sarc} would not comprise a major portion of the DNA transcribed from AS virus RNA by reverse transcriptase (cf. Table 1). Consequently, effective separation of cDNA_{sarc} from DNA hybridized to td RNA required exceptional

TABLE 1
Purification of cDNA_{sarc} for Pr-C AS virus

Step in purification	DNA recovered as Single-strand (cts/min)	Hybrid (cts/min)	Recovery of DNA in step (%)	Cumulative recovery of DNA (%)
(a) Single-stranded DNA (4.2 µg, 84 × 10 ⁶ cts/min) hybridized with 10 µg 70 S RNA of Pr-C AS virus; final <i>C_tt</i> = 100	27 × 10 ⁶	30 × 10 ⁶	68	68
(b) DNA recovered as hybrid in step (a) hybridized to 40 µg 70 S RNA of td Pr-C AS virus; final <i>C_tt</i> = 200	5 × 10 ⁶	13 × 10 ⁶	60	41
(c) Single-stranded DNA from step (b) rechromatographed on hydroxylapatite	4 × 10 ⁶	---	80	33
(d) Single-stranded DNA from step (c) hybridized with 10 µg 70 S RNA of td Pr-C AS virus; final <i>C_tt</i> = 100	2.5 × 10 ⁶	7 × 10 ⁴	63	21
(e) Single-stranded DNA from step (d) hybridized with 8 µg 70 S RNA from Pr-C AS virus; final <i>C_tt</i> = 25	6 × 10 ⁵	1.1 × 10 ⁶	68	14

chromatographic resolution. We achieved the necessary fractionation by eluting single-stranded DNA from hydroxylapatite with a shallow gradient of sodium phosphate, followed by elution of viral RNA and RNA/DNA hybrids with a wash of 0.4 M-sodium phosphate; the resolution provided by this procedure is illustrated in Figure 2. No effort was made to separate viral RNA from hybrids; this is a difficult if not impossible separation to accomplish in the present instance because the chromatographic behavior of the hybrids is determined by the RNA constituent, whose mass exceeds the mass of DNA constituents by a factor of at least ten (Fanshier *et al.*, 1971).

(ii) *Selection of virus-specific DNA and removal of reiterated nucleotide sequences*

Prior to selection of cDNA_{sarc}, the entire preparation of virus-specific single-stranded DNA was annealed with a limiting amount of AS virus 70 S RNA (RNA/DNA ratio 2), and the DNA which failed to hybridize to RNA was removed by chromatography on hydroxylapatite (step (a), Fig. 3). This procedure served two purposes.

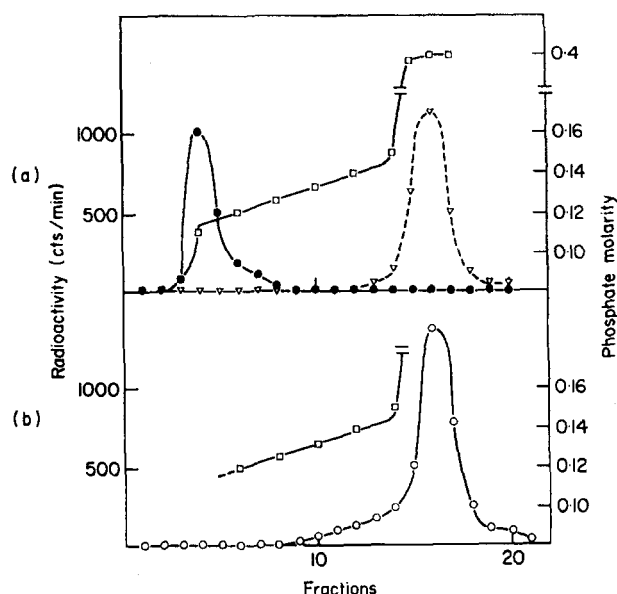


FIG. 2. Fractionation of nucleic acids by chromatography on hydroxylapatite.

Chromatography on columns of hydroxylapatite was carried out as described under Materials and Methods. (a) Fractionation of single-stranded [^3H]DNA prepared with detergent-activated virions of AS virus (—●—●—) and 70 S [^{32}P]RNA from Pr-C AS virus (---▽---). (b) Elution of RNA/DNA hybrid (—○—○—), formed by reacting virus-specific single-stranded [^3H]DNA with a large excess of unlabeled 70 S RNA of Pr-C AS virus. In both panels, the gradients of sodium phosphate are depicted as (—□—□—).

First, it ensured the virus-specific nature of the DNA by discarding transcripts from cellular RNA included in viral particles (Garapin *et al.*, 1973). Second, by limiting the amount of RNA available for hybridization, reiterated nucleotide sequences which are abundant in the initial enzymatic product (Garapin *et al.*, 1973) were preferentially discarded; this simplified the logistics of subsequent steps in the preparation of $\text{cDNA}_{\text{sarc}}$.

(iii) Stabilization of RNA/DNA hybrids during chromatography on hydroxylapatite

Initial preparations of $\text{cDNA}_{\text{sarc}}$ were purified by fractionation with sodium phosphate on hydroxylapatite following hybridization with td 70 S RNA. The putative $\text{cDNA}_{\text{sarc}}$ failed to hybridize to td RNA when retested on hydroxylapatite (Fig. 4(a)), yet hybridized as much as 60% when tested with S1 nuclease. We speculated that the hybrids detected by the nuclease were disrupted by the standard conditions for loading hydroxylapatite (0.01 M-sodium phosphate, 60°C) and tested this hypothesis by adding 0.6 M-NaCl to all of the solutions used for chromatography. The resolution achieved by chromatography was unaffected by the addition of 0.6 M-NaCl to the elution buffers, but the procedure now detected hybrids in amounts approximately equivalent to those detected by hydrolysis with S1 nuclease (Fig. 4(b)). Consequently, all solutions were supplemented with 0.6 M-NaCl for the preparative chromatographies which followed hybridization of DNA to td RNA (Fig. 3, steps (b), (c) and (d)).

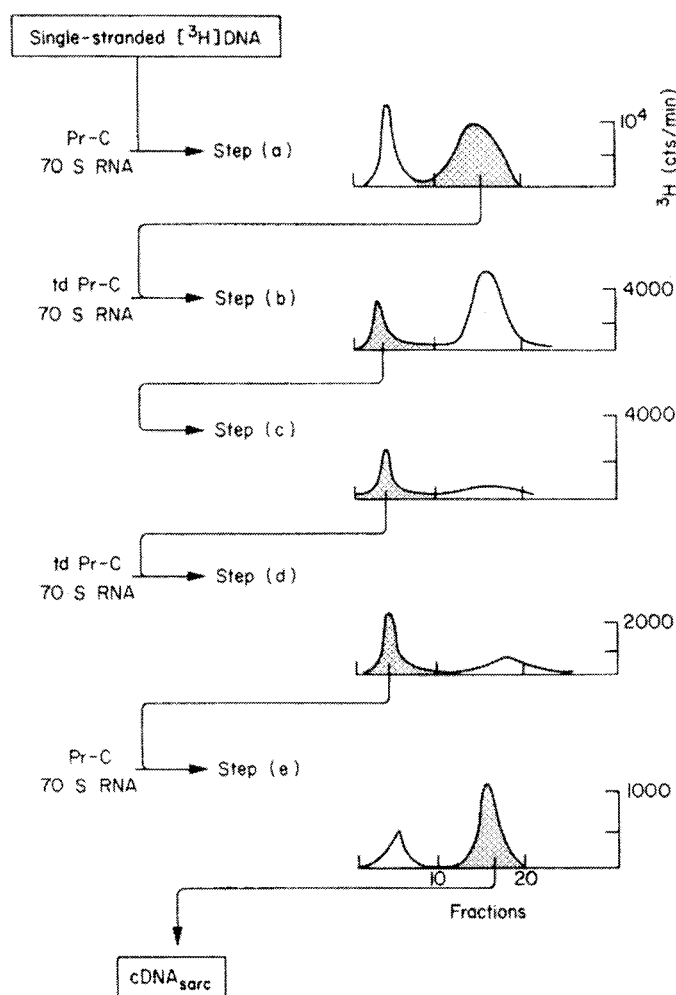


FIG. 3. Purification of $cDNA_{sarc}$: schematic summary.

Step (a). Single-stranded [3H]DNA synthesized with Pr-C AS virus and isolated as described under Materials and Methods was hybridized to Pr-C AS virus 70 S RNA. RNA/DNA hybrids were purified by fractionation on hydroxylapatite and the DNA recovered after hydrolysis of RNA with alkali.

Step (b). The DNA recovered from hybrid in step (a) was annealed to a large excess of 70 S RNA from td Pr-C AS virus; the DNA remaining unhybridized was isolated by fractionation on hydroxylapatite in 0.6 M-NaCl.

Step (c). Unhybridized DNA recovered in step (b) was rechromatographed on hydroxylapatite in the presence of 0.6 M-NaCl.

Step (d). Single-stranded DNA recovered from the column in step (c) was annealed with a large excess of 70 S RNA from td Pr-C AS virus, then chromatographed on hydroxylapatite in the presence of 0.6 M-NaCl.

Step (e). The unhybridized (single-stranded) DNA recovered from hydroxylapatite in step (d) was annealed with a large excess of Pr-C AS virus 70 S RNA as a final selection for virus-specific nucleotide sequences. RNA/DNA hybrids were isolated by chromatography on hydroxylapatite, and the DNA recovered from the hybrids by hydrolysis with alkali was designated $cDNA_{sarc}$.

Experimental details for these steps are provided in Table 1. Stippled areas represent material pooled for subsequent steps.

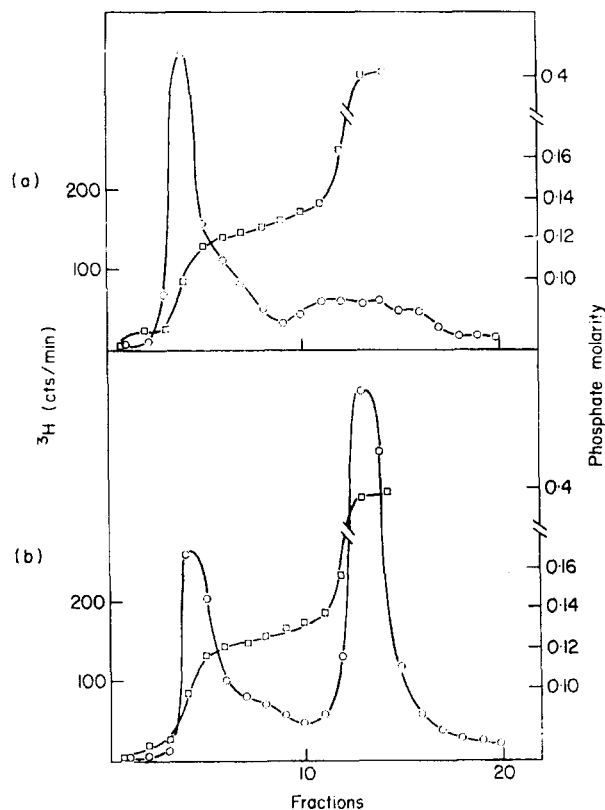


FIG. 4. Stabilization of RNA/DNA hybrids for chromatography on hydroxylapatite.

Single-stranded [^3H]DNA (10 ng, 2×10^6 cts/min) synthesized with Pr-C AS virus and isolated as described under Materials and Methods was hybridized to unlabeled 70 S RNA of td Pr-C AS virus (10 μg ; final $C_{\text{r}}t = 200$ mol·s/l). The unhybridized DNA was isolated by chromatography on hydroxylapatite and rehybridized to 70 S RNA of td Pr-C AS virus; 60% of the DNA was resistant to hydrolysis by S1 nuclease at the conclusion of the hybridization (unhybridized DNA was 7% resistant to hydrolysis). The nucleic acids were then fractionated on hydroxylapatite, using either the conventional phosphate buffers (a) or buffers supplemented with 0.6 M NaCl (b). —○—○—, ^3H radioactivity (cts/min); —□—□—, gradients of sodium phosphate.

(b) Preparation of $c\text{DNA}_{\text{sarc}}$ for Pr-C AS virus

Following the protocol explained above and outlined in Figure 3 and Table 1, we isolated about 55 ng ($\sim 1.1 \times 10^6$ cts/min) of $c\text{DNA}_{\text{sarc}}$ from 4.2 μg ($\sim 84 \times 10^6$ cts/min) of single-stranded DNA synthesized with Pr-C AS virus. Two steps in the procedure proved unnecessary and have been omitted from subsequent preparations. First, the rechromatography on hydroxylapatite at step (c) did not effect significant further purification of the single-stranded DNA. Second, repeated hybridization of DNA with td RNA (step (d)) proved superfluous; use of a sufficient excess of td RNA in the first hybridization (step (b)) removed all virus-specific deoxynucleotide sequences not represented in the deleted region of the viral genome. In the preparation outlined, about 5% of the complementary DNA annealed with td RNA and 77% annealed with AS virus RNA after step (b). Therefore, the subsequent steps made only modest improvements in the purity of $c\text{DNA}_{\text{sarc}}$ (cf. Fig. 6), but they caused considerable losses (cf. Table 1).

About 1.3% of the single-stranded complementary DNA synthesized with Pr-C AS virus was recovered as $\text{cDNA}_{\text{sarc}}$. By correction for the cumulative losses of DNA during the course of the procedure (see Table 1), we compute that $\text{cDNA}_{\text{sarc}}$ comprised approximately 9% of the original single-stranded enzymatic product. This single preparation of $\text{cDNA}_{\text{sarc}}$ was used in all of the studies which follow.

(c) *Size of $\text{cDNA}_{\text{sarc}}$*

The molecular weights of [^3H]cDNA $_{\text{sarc}}$ for Pr-C AS virus and [^{32}P]cDNA $_{\text{B77}}$ were compared by rate-zonal centrifugation (Fig. 5). The two preparations of DNA sedimented similarly, with average sedimentation coefficients (in 0.1 M-NaCl, pH 7)

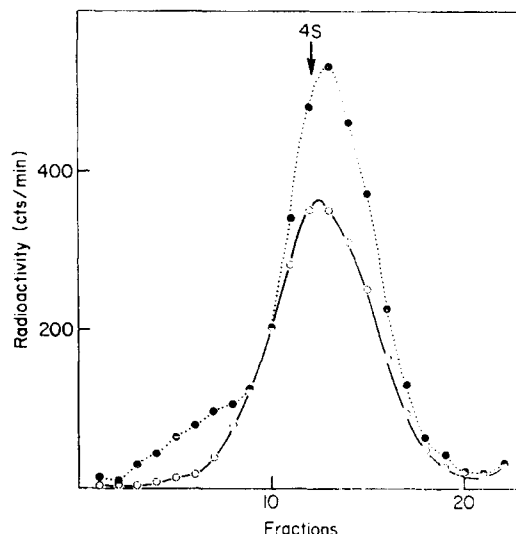


FIG. 5. Analysis of virus-specific DNAs by rate-zonal centrifugation.

[^3H]cDNA $_{\text{sarc}}$ (3000 cts/min) of Pr-C AS virus and [^{32}P]cDNA $_{\text{B77}}$ (5000 cts/min) were sedimented through a gradient of 15% to 30% (w/v) sucrose containing 0.1 M-NaCl, 0.001 M-EDTA, 0.02 M-Tris-HCl (pH 7.4). Centrifugation was carried out in a Spinco SW65 rotor (17 h, 64,000 revs/min, 4°C). Gradients were fractionated and analyzed for acid-precipitable radioactivity (Bishop *et al.*, 1970). Sedimentation was from right to left.

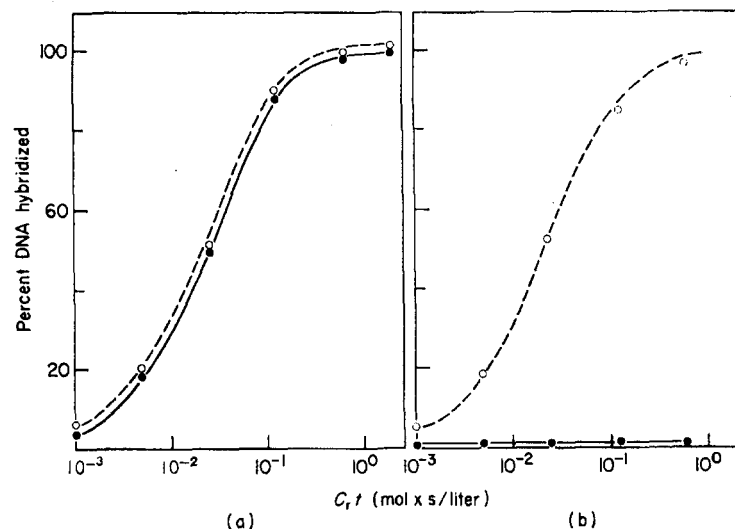
...●...●..., ^{32}P (cts/min); —○—○—, ^3H (cts/min).

of about 4 S. This finding conforms to previous studies on unselected reverse transcriptase product (Temin & Baltimore, 1972) and indicates that molecular size was not a major determinant in the selection of $\text{cDNA}_{\text{sarc}}$.

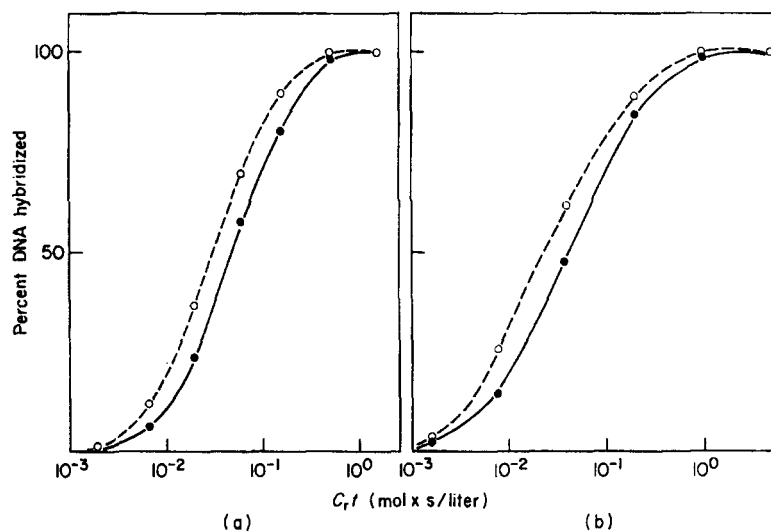
(d) *Specificity (purity) of $\text{cDNA}_{\text{sarc}}$*

The specificity of $\text{cDNA}_{\text{sarc}}$ was examined by hybridization with 70 S RNAs from cloned Pr-C AS virus and td Pr-C AS virus (Fig. 6); [^{32}P]cDNA $_{\text{B77}}$ was included in the reactions to serve as an internal standard for hybridization. Both complementary DNAs hybridized completely with Pr-C AS virus RNA with identical kinetics. This is the expected result, since the reactions are driven by a vast excess of RNA and are not appreciably affected by the complexities of the two populations of DNA. Only the cDNA $_{\text{B77}}$ reacted with RNA from the td variant (Fig. 6(b)).

The preparation of $\text{cDNA}_{\text{sarc}}$ was designed to ensure the isolation of virus-specific

FIG. 6. Specificity of $cDNA_{sarc}$.

The 70 S RNAs of Pr-C AS virus (a) and td Pr-C AS virus (b) were hybridized with $[^{32}P]cDNA_{B77}$ (—○—○—) and $[^3H]cDNA_{sarc}$ of Pr-C AS virus (—●—●—) in 20 μ l of annealing buffer containing 0.9 M-NaCl. Individual reactions contained 0.02 ng (1000 cts/min) of $[^{32}P]$ DNA, 0.03 ng (600 cts/min) of $[^3H]$ DNA, and amounts of RNA varying from 0.1 ng to 300 ng; incubations were at 68°C for 4 h. Extent of hybridization was measured by hydrolysis with S1 nuclease. Resistance of unannealed complementary DNAs to S1 nuclease was less than 3%.

FIG. 7. Hybridization of $cDNA_{sarc}$ with native RNA and genome subunits of Pr-C AS virus.

The 70 S RNA of Pr-C AS virus was purified and a portion denatured for the isolation of 35 S subunits by rate-zonal centrifugation as described under Materials and Methods. The virus used in this work contained an appreciable amount ($\sim 50\%$ of virus particles) of td mutants (unpublished data). Native RNA (0.0004 μ g to 0.3 μ g per sample) and subunits (0.0003 μ g to 1 μ g per sample) were reacted separately with mixtures of $[^3P]cDNA_{sarc}$ (—●—●—) (800 cts/min and 0.04 ng per sample) and $[^{32}P]cDNA_{B77}$ (—○—○—) (800 cts/min and 0.02 ng per sample) in annealing buffer containing 0.6 M-NaCl at 68°C. The extent of hybridization was measured by hydrolysis with S1 nuclease.

(a) Native RNA; (b) RNA subunits.

nucleotide sequences; two observations indicate that this was in fact accomplished. First, cDNA_{sarc} and cDNA_{B77} hybridized with RNA from cloned Pr-C AS virus with identical kinetics (Fig. 6(a)); this would not occur if cDNA_{sarc} were transcribed from a minor contaminant of RNA in the virus. Second, we denatured the 70 S RNA of Pr-C AS virus, isolated 35 S subunits of the viral genome by rate-zonal centrifugation, and found that these hybridized at the same rates as the native 70 S RNA with cDNA_{sarc} and cDNA_{B77} (Fig. 7). The two DNAs hybridized at slightly different rates; we attribute this to the presence of td genomes in the viral RNA (see Discussion). We conclude that cDNA_{sarc} contains virus-specific nucleotide sequences present in the genome of Pr-C AS virus but deleted from the RNA of a td variant.

(e) *Genetic complexity of the nucleotide sequences in cDNA_{sarc}*

We used molecular hybridization to determine what fraction of the viral genome was represented in cDNA_{sarc} and whether the representation was uniform. ³²P-labeled 70 S RNA of cloned Pr-C AS virus was hybridized with cDNA_{sarc} under two sets of conditions. First, the hybridization was carried out for various periods of time with equivalent amounts of RNA and DNA. This reaction was complete when 16% of the RNA had hybridized (Fig. 8(a)); hence, the cDNA_{sarc} represents approximately

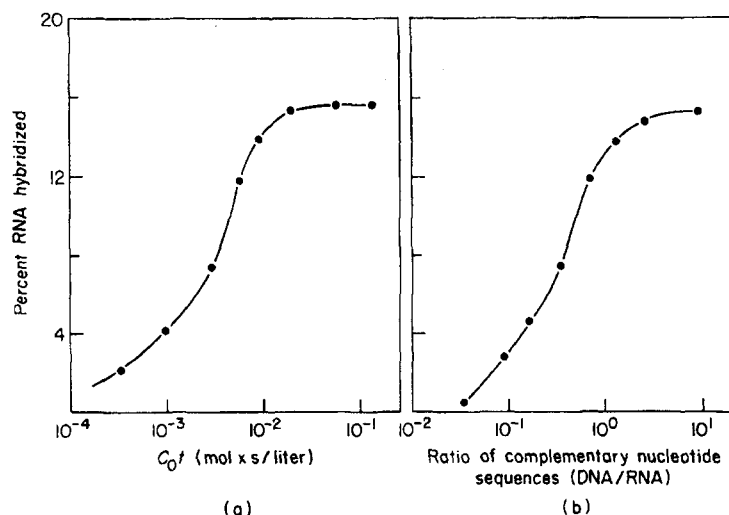


Fig. 8. Genetic complexity of the nucleotide sequences in cDNA_{sarc}.

³²P-labeled 70 S RNA ($\sim 2 \times 10^7$ cts/min per μ g) of cloned Pr-C AS virus was hybridized with [³H]cDNA_{sarc} at 68°C in annealing buffer containing 0.6 M-NaCl. Extent of hybridization of the RNA was measured by hydrolysis with RNase A as described under Materials and Methods. The data have been corrected for the intrinsic resistance of viral RNA to hydrolysis by RNase ($\sim 2\%$). Two sets of conditions were employed, as follows. (a) The cDNA_{sarc} and [³²P]RNA were mixed at a constant ratio (2000 cts/min and 0.1 ng of RNA, 2000 cts/min and 0.1 ng of cDNA_{sarc}) in 4 μ l and incubated at 68°C to the indicated values of C_0t (computed from the concentration of the DNA alone, with incubation periods ranging from 10 min to 40 h); the 2 highest values of C_0t were obtained by increasing the amount of complementary DNA in the reactions to 1 ng.

(b) A constant amount of [³²P]RNA (20,000 cts/min and 1 ng) was incubated with different amounts of cDNA_{sarc} (~ 0.03 to 1.5 ng) for 40 h; the value of C_0t ($\sim 6 \times 10^{-2}$ mol·s/l computed from the concentration of DNA (25 ng/ml)) was held constant by varying the reaction volume. Two low ratios of complementary DNA/RNA (0.05 and 0.09) were achieved by increasing the amount of RNA in the reactions to 200,000 cts/min (10 ng) and using appropriate amounts of complementary DNA; this variation was necessary to maintain a practicable reaction volume.

16% of the Pr-C AS virus genome and was actually in sevenfold excess of complementary ribonucleotide sequences in the hybridization reaction. Second, we performed hybridizations in which the value of C_0t was held constant ($\sim 5 \times 10^{-2}$ mol·s/l, chosen on the basis of the data in Fig. 8(a)) and the ratio of cDNA_{sarc} to Pr-C AS virus 70 S RNA was varied over a wide range (Fig. 8(b)). As before, a maximum of 16% of the viral RNA was hybridized; saturation was reached at a ratio of complementary nucleotide sequences (DNA/RNA) of approximately 3:1.

We conclude that cDNA_{sarc} is a relatively uniform transcript from 16% of the nucleotide sequences in the genome of Pr-C AS virus; this value conforms to previous estimates of the size of the deletion in the strain of td Pr-C AS virus used in our experiments (Duesberg & Vogt, 1973a; Lai *et al.*, 1973) and indicates that cDNA_{sarc} represents the entire deletion. Since the unit genome of AS virus is thought to contain 10,000 nucleotides (Beemon *et al.*, 1974; Billeter *et al.*, 1974), the genetic complexity of cDNA_{sarc} is about 1600 nucleotides.

(f) *Nucleotide sequences of cDNA_{sarc} in other strains of RNA tumor viruses*

The cDNA_{sarc} from Pr-C AS virus hybridized completely with genome RNAs from six strains of AS virus (Table 2), including three strains (Carr-Zilber, Bryan and Schmidt-Ruppin) believed to be derived from the original isolate of P. Rous, two

TABLE 2
Nucleotide sequences of cDNA_{sarc} distribution among different strains of RNA tumor viruses

Viral RNA	Hybridization to:†	
	[³² P]cDNA _{B77} (%)	[³ H]cDNA _{sarc} (Pr-C AS virus) (%)
Pr-C AS virus	95	100
B77-C AS virus	100	95
Carr-Zilber AS virus	95	100
Schmidt-Ruppin AS virus	100	93
Bryan AS virus	97	94
Fujinami AS virus	94	92
td Pr-C AS virus	95	1
td B77-C AS virus	100	1
Rous-associated virus-0	72	1
Rous-associated virus-2	80	0
Rous-associated virus-6	82	0
Rous-associated virus-50	80	1
Avian myeloblastosis virus	81	2
Ring-necked pheasant virus	84	1
Murine sarcoma-leukosis virus (Moloney)	nt‡	0
Feline sarcoma-leukosis virus (Rickard)	nt	0
Murine mammary tumor virus (RIII)	nt	0
Visna virus	nt	0

† [³H]cDNA_{sarc} of Pr-C AS virus and [³²P]cDNA_{B77} were hybridized with 70 S RNAs obtained from the indicated viruses; incubation of 600 to 1000 cts/min of each complementary DNA was carried out in 0.6 M-NaCl at 68°C to a final C_0t of at least 50 mol·s/l (the C_0t_1 for 70 S RNA is $\sim 2 \times 10^{-2}$ mol·s/l; see Taylor *et al.*, 1974). The extent of hybridization was measured by hydrolysis with S1 nuclease (Leong *et al.*, 1972); resistance of unhybridized complementary DNAs to S1 nuclease was less than 3%.

‡ nt denotes not tested. The RNAs from the mammalian viruses were tested with homologous complementary DNAs and found to hybridize appropriately (data not shown).

independent isolates (strains Bratislava 77 and Fujinami), and Prague strain which is of uncertain lineage (Morgan & Traub, 1964). By contrast, there was no detectable hybridization between $cDNA_{sarc}$ and RNA from td variants of AS virus, avian leukosis viruses and an endogenous virus of chickens, Rous-associated virus-0; none of these viruses can induce sarcomas in animals or transform fibroblasts in culture. In addition, $cDNA_{sarc}$ failed to hybridize with the RNAs of tumor viruses from other species (murine sarcoma-leukosis virus, feline sarcoma-leukosis virus and murine mammary tumor virus) and of the slow virus visna. $[^{32}P]cDNA_{B77}$ hybridized completely with RNA from all of the AS viruses tested and extensively (70 to 85%) with RNA from the non-transforming avian viruses.

Hybrids between Pr-C AS virus $cDNA_{sarc}$ and RNA from either Pr-C AS virus or B77-C AS virus were indistinguishable by thermal denaturation (Fig. 9): the same

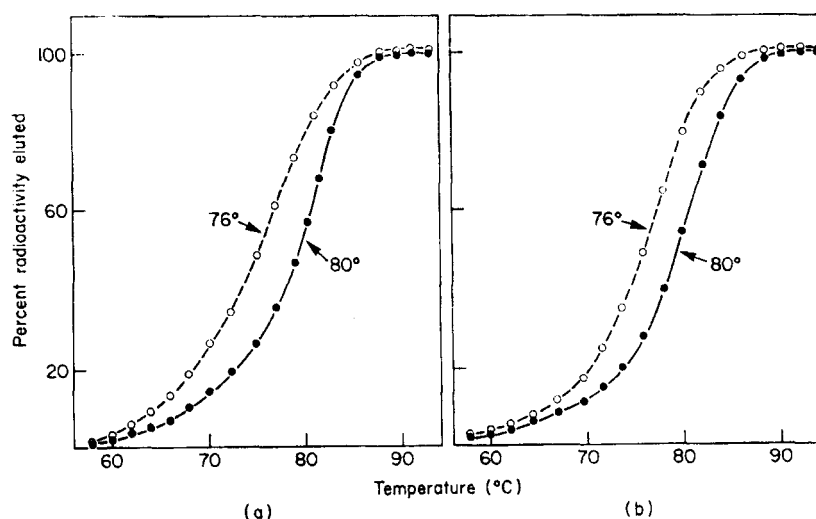


FIG. 9. Thermal denaturation of RNA/DNA hybrids on hydroxylapatite.

RNA/DNA hybrids were formed and their thermal denaturation analyzed by elution from hydroxylapatite as described under Materials and Methods. Data are presented as cumulative elution of $[^3H]cDNA_{sarc}$ (—●—●—) and $[^{32}P]cDNA_{B77}$ (---○---○---) with increasing temperature. (a) Hybrids with 70 S RNA of Pr-C AS virus. (b) Hybrids with 70 S RNA of B77-C AS virus.

was true for hybrids formed with $cDNA_{B77}$. Hence, there is little difference in the nucleotide sequences homologous to either of the DNAs in the two strains of AS viruses. Although hybrids with $cDNA_{sarc}$ had a t_m 5 degrees higher than that for the hybrids with $cDNA_{B77}$, the uneven representation of the genome in $cDNA_{B77}$ makes this difference difficult to interpret.

(g) Preparation of $cDNA_{sarc}$ for B77-C AS virus

We have used the methods described above to prepare $cDNA_{sarc}$ for B77-C AS virus; the results were comparable to those obtained with Pr-C AS virus (data not shown). The $cDNA_{sarc}$ for B77-C AS virus represented at least 10% of the nucleotide sequences in the B77-C genome when tested as described for Figure 8, hybridized completely with all of the AS virus RNAs listed in Table 1, and failed to hybridize with RNAs from td variants, avian leukosis viruses, and mammalian C-type viruses.

Analysis by thermal denaturation (as described for Fig. 9) revealed no detectable mismatching in hybrids between cDNA_{sarc} for B77-C AS virus and RNA from Pr-C AS virus.

4. Discussion

(a) *Purification of cDNA_{sarc}*

We have developed a method for the purification of DNA complementary to a region deleted from the genome of a mutant virus. We have employed an avian sarcoma virus and a transformation-defective deletion mutant because the deleted sequences are of obvious biological interest. The general strategy, however, is potentially applicable to several other situations: preparation of DNA specific for deletions present in other avian RNA tumor virus mutants (e.g. certain glycoprotein (Kawai & Hanafusa, 1973) and reverse transcriptase (Hanafusa *et al.*, 1972) mutants); DNA specific for sequences found exclusively in one of two related RNA tumor viruses (e.g. sequences present in murine sarcoma virus but absent from murine leukemia virus (Stephenson & Aaronson, 1971)); or DNA specific for any virus or cell when there is a closely related genome lacking the relevant sequences. A similar approach has been employed by Baxt & Spiegelman (1973) to enrich the DNA synthesized by human leukemic cell polymerases for nucleotide sequences specific for leukemic as opposed to normal cell DNA.

The scheme outlined in Figure 1 will lead to the co-purification of cDNA_{sarc} and any contaminating DNA present in the initial transcript unless a preparative annealing to 70 S RNA from sarcoma virus (step (a)) is included. We have enlarged the benefits of this step by performing the annealing at a relatively low ratio of sarcoma virus RNA to DNA. As a result, portions of DNA copied at high frequency from regions outside the deletion remain unannealed and are discarded with the small amount of DNA copied from cellular nucleic acids associated with the virus particle (Garapin *et al.*, 1973). Since we have used 70 S RNA for the selection procedures, it could be argued that we have purified DNA copied from some 70 S-associated 4 S or 5 S RNA (Erikson & Erikson, 1971; Faras *et al.*, 1973) found only in the transforming virus. We have excluded this possibility by showing that the purified cDNA_{sarc} anneals to 35 S subunits of the viral genome which have been dissociated from low molecular weight components of cellular origin (Fig. 7); in addition, we have shown that cDNA_{sarc} is a homogeneous transcript of 16% of 70 S RNA (Fig. 8). Such a large component of 70 S RNA cannot be accounted for by cellular RNAs associated with the 70 S complex.

We estimate that cDNA_{sarc} constituted almost 10% of the initial transcript from the Pr-C AS virus genome; other portions of the AS virus genome may be represented more or less frequently in the enzymatic product (Garapin *et al.*, 1973). The relative frequency of transcription from the region of cDNA_{sarc} suggests to us that this region is reasonably close to the site for initiation of transcription from the AS virus genome. This suggestion raises a paradox, since the site for binding of the 4 S RNA molecule which primes DNA synthesis *in vitro* has been located at or near the 5' terminus of AS virus (Taylor & Illmensee, 1975), whereas the nucleotide sequences of cDNA_{sarc} are close to the 3' terminus (Wang & Duesberg, 1974; also, unpublished observations of J. Tal and the authors). Possibly the secondary structure of the viral genome juxtaposes the site of initiation of DNA synthesis and the cDNA_{sarc} region, permitting frequent transcription of cDNA_{sarc}.

(b) *Genetic specificity and complexity of cDNA_{sarc}*

Results of recent genetic analyses indicate that AS virus may have a single gene responsible for neoplastic transformation of fibroblasts (Wyke *et al.*, 1974). The genetic complexity of cDNA_{sarc} (~1600 nucleotides) is sufficient to represent an entire cistron and could reasonably account for the entirety of the transforming gene. It is also possible that the deletion by which cDNA_{sarc} was defined extends into other areas of the viral genome or represents nucleotide sequences required for a transforming function other than that defined by available temperature-sensitive mutants (Wyke *et al.*, 1974); on the other hand, td variants have no identified physiological deficit other than the defect in transformation and even retain some oncogenic potential in the form of an ability to induce leukosis (Biggs *et al.*, 1973). In addition, neither td variants nor avian leukosis viruses complement any of the known temperature-sensitive mutants for transformation (Wyke & Linial, 1973; Wyke *et al.*, 1974; Bernstein, McCormick & Martin, unpublished results), suggesting that they bear a deletion in the gene defined by such mutants.

The genomes of avian RNA tumor viruses are apparently composed of two or more identical subunits, each containing approximately 10,000 nucleotides (Beemon *et al.*, 1974; Billeter *et al.*, 1974); hence the genomes are considered genetically polyploid (Vogt, 1973; Duesberg & Vogt, 1973b). The identical rates of hybridization of [³²P]cDNA_{B77} and [³H]cDNA_{sarc} to RNA from a cloned AS virus (Fig. 6) indicate that the nucleotide sequences of cDNA_{sarc} are present in 70 S RNA in the same frequency as other gene sequences; it follows that the deleted nucleotide sequences are the same for each subunit, as has been concluded from other evidence (Duesberg & Vogt, 1970, 1973b).

RNA from uncloned (or repeatedly passaged) AS virus can give different rates of hybridization with cDNA_{B77} and cDNA_{sarc}; for example, there was a twofold difference between the rates of the two reactions illustrated in Figure 7. These differences are attributable to the fact that a portion of the viral RNA is composed of td genomes which can hybridize with the unfractionated cDNA_{B77} but not with cDNA_{sarc}. We have exploited these rate differences to measure the amounts of td variants in virus stocks (unpublished results).

(c) *Homology of nucleotide sequences in the genomes of avian RNA tumor viruses*

The strain of B77-C AS virus used to prepare single-stranded [³²P]DNA was comprised mainly (~90%) of td variants. Consequently, the DNA represents nucleotide sequences shared by td genomes and genomes of the parental AS virus; this accounts for the complete reaction between the [³²P]cDNA_{B77} and the RNAs of td strains (Table 2). The [³²P]cDNA_{B77} also reacted completely with RNAs from heterologous strains of AS virus (Table 2) and extensively (albeit not completely) with RNAs from several strains of avian leukosis viruses (Table 2); hybrids between the [³²P]-cDNA_{B77} and RNA from either B77-C AS virus (the homologous strain) or Pr-C AS virus (a heterologous strain) denatured in an almost identical fashion (Fig. 9). These observations conform to previous reports that extensive genetic homology exists among the avian RNA tumor viruses; molecular hybridization has revealed no detectable differences among the various sarcoma viruses and only moderate differences between the sarcoma and leukosis viruses (Kang & Temin, 1973; Wright & Neiman, 1974).

The close homology among the genomes of different AS viruses is mirrored in the studies with cDNA_{sarc}, which can hybridize completely with RNA from six strains of AS virus, including three independent isolates (Table 2). Moreover, analysis by thermal denaturation revealed no detectable divergence in the nucleotide sequences homologous to cDNA_{sarc} in Pr-C and B77-C AS viruses (Fig. 9). These data suggest closely related or common lineages for the genes of AS viruses, including the gene(s) responsible for neoplastic transformation of fibroblasts. However, chemical analyses capable of detecting changes in single nucleotides have demonstrated differences among the transforming genes of several strains of AS virus (Beemon *et al.*, 1974); the extent of these differences cannot be assessed from available data, but our results with thermal denaturation suggest less than 1% mismatching of Pr-C cDNA_{sarc} annealed to B77-C RNA (Ullman & McCarthy, 1973).

The physical homology of transforming genes in different strains of AS virus is not apparent in the genetic interactions of these viruses. Temperature-sensitive mutants of AS virus conditional for transformation can recombine genetically (see Wyke *et al.*, 1974) only if the coinfecting mutants are derived from the same parental strain of AS virus (Wyke, 1973); hence, the physical homology between the interacting transforming genes does not suffice to permit genetic crossing-over (Beemon *et al.*, 1974).

(d) General utility of cDNA_{sarc}

Our success in preparing cDNA_{sarc} represents further confirmation of previous demonstrations that td variants of AS viruses are deletion mutants (Duesberg & Vogt, 1970, 1973b; Lai *et al.*, 1973; Neiman *et al.*, 1974). The nucleotide sequences in cDNA_{sarc} represent most or all of the deletion for the td strain employed by us and must therefore include genetic information required for neoplastic transformation of fibroblasts. Hence, cDNA_{sarc} should facilitate the analysis of several problems central to the study of RNA tumor viruses, including the following. (1) Are genes homologous or related to the transforming gene(s) of AS viruses present in uninfected cells and, if so, under what circumstances are they expressed? (2) Do the transforming genes of sarcoma (and carcinoma) viruses from different species share nucleotide sequences? Our limited survey to date suggests that they do not. (3) How is the expression of transforming gene(s) regulated in infected cells?

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